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Molecular phylogeny of the endemic East African flightless grasshoppers *Altiusambilla* Jago, *Usambilla* (Sjöstedt) and *Rhainopomma* Jago (Orthoptera: Acridoidea: Lentulidae)

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Abstract. A molecular phylogeny of endemic flightless grasshoppers is presented for the three Lentulidae genera *Altiusambilla* Jago, 1981, *Usambilla* Sjöstedt, 1909 and *Rhainopomma* Jago, 1981 based on DNA sequences (16S rRNA locus). Parsimony, distance and likelihood reconstructions were performed using different assumptions on sequence evolution. The generated phylogenies agree in almost all parts of the calculated trees and support the monophyly of the observed genera. It was shown that *Usambilla* and *Rhainopomma* are more closely related to each other, *Altiusambilla* being a separate clade. However, the investigated East African lentulid genera are clearly separated from South African taxa, underlining the monophyly of East African genera. *Usambilla olivacea* is re-established. Populations of *Rhainopomma montanum* from the Taita Hills of Kenya and from the West Usambara mountains of Tanzania are two separate species not closely related to each other. *Rhainopomma* samples from the North Pare mountains of Tanzania belong to a hitherto undescribed species.

Introduction

The family Lentulidae is a comparatively small family, with the two subfamilies Lentulinae (80 species in 21 genera) and the purely South African distributed subfamily Shelforditinae with mostly monotypic genera (ten genera and 13 species).

Species of the investigated genera (Fig. 1) *Altiusambilla* Jago, 1981, *Rhainopomma* Jago, 1981 and *Usambilla* (Sjöstedt, 1909) mostly occur on currently climatically isolated high mountains or mountain ranges, such as the so-called Eastern Arc mountains (e.g. Pare mountains, Usambara mountains, Taita Hills) and inland volcanoes (Fig. 2). The Eastern Arcs were ranked among the ten most important hotspots for endemism by Myers *et al*. (2000). These mountain ranges harbour huge evergreen rainforests due to the westward winds that constantly bring moisture from the Indian Ocean (Hamilton, 1989). The increased biodiversity and an accumulation of endemic species in this region are comparable with Madagascar (Pócs, 1998). Some authors even described this area as the 'Galápagos of Africa' (Rodgers & Homewood, 1982; Kingdon, 1990). In contrast to the old mountain formations of the Eastern Arcs (estimated age approximately 30 My; Burgess *et al*., 1998), the volcanoes Kilimanjaro (1–3 My), Mount Meru (2 My) and Mount Kenya (5 My) are much younger (Marek, 2001).

To illuminate the speciation processes of flightless East African Saltatoria, species of the genera *Usambilla*, *Altiusambilla* and *Rhainopomma* were investigated. *Usambilla* and allies contain 33% of all known Lentulinae species. Their morphology and distribution patterns show that these species are closely related and in a recent radiation. Approximately half the 21 Lentulinae genera are restricted to southern Africa, few occur in tropical South Africa. Almost 70% of all species of the family Lentulidae only occur in southern Africa, with their diversity centre in the Cape Province of South Africa. *Mecostibus* is one of the most species-rich genera of Lentulidae, with 13 species, and

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Fig. 1. Males and females of *Rhainopomma*, *Usambilla* and *Altiusambilla*. A, *Rhainopomma usambaricum*, male (East Usambara mountains, Tanzania); B, *Usambilla emaliensis*, male (Machakos, Kenya); C, *Alitusambilla modicicrus*, male (Mount Kilimanjaro, Tanzania); D, *Rhainopomma usambaricum*, female; E, *Usambilla* sp. (Mount Hanang, Tanzania), female; F, *Altiusambilla modicicrus*, female.

shows the widest distribution in tropical South, North-east, West-central and East Africa. Genera investigated in this paper have their main distribution in tropical East Africa.

Jago (1981) revised the genus *Usambilla* and its allies. He erected the four new genera *Chromousambilla*, *Microusambilla*, *Altiusambilla* and *Rhainopomma* on the basis of male genitalic morphology. *Chromousambilla* was based on *Adolfia latestriata* Ramme and four additional species were described by Jago (1981). This genus only occurs in north-west Zambia and southern Tanzania. The monotypic *Microusambilla* is restricted to Zimbabwe and was erected for *U. cylindricollis* Ramme. The monotypic genus *Altiusambilla*

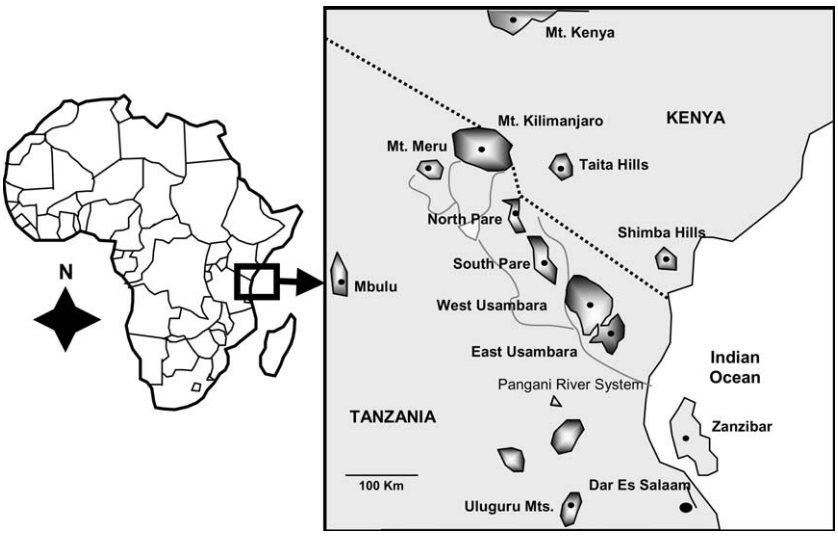

Fig. 2. Map of East Africa in the area of the Eastern Arcs and inland volcanoes of Kenya and Tanzania.

Table 1. Species of the genus *Rhainopomma* Jago.

Original description	Jago 1981	Distribution
Adolfia usambarica Ramme	*R. usambaricum* (Ramme)	Tanzania, East Usambara; Kenya, Shimba Hills
Usambilla montana Kevan	*R. montanum* (Kevan)	Kenya, Taita Hills; Tanzania, West Usambara, South Pare
	R. magnificum Jago	Tanzania, South Pare
	R. nguruense Jago	Tanzania, Nguru Mts.
	R. wapugu Jago	Tanzania, Pugu Hills

was erected for *Lentula modicicrus* Karsch from Mount Kilimanjaro. The type species of *Rhainopomma* is *Adolfia usambarica* Ramme from the East Usambara mountains of Tanzania. Jago (1981) placed *U. montana* Kevan into *Rhainopomma* and described three new species (Table 1).

Jago (1981) transferred species from *Lentula* and *Adolfia* into *Usambilla*, erected on *U. olivacea* Sjöstedt from the West Usambara mountains of Tanzania, and described five additional species and two new subspecies (Table 2). Most species occur in East Africa.

He noted that, based on genitalic morphology, *Altiusambilla* has affinities to *Rhainopomma* and especially to *Mecostibus*, but also stated that morphological similarities between *Rhainopomma* and *Altiusambilla* may be due to convergent evolution.

We analysed the phylogeny of East African species to understand the evolution of regional endemism and to determine the fit of these results with the existing taxonomy.

Materials and methods

Samples

Lentulid specimens were collected in the area of the East African countries Kenya and Tanzania (Table 3, Fig. 2). Fresh specimens were fixed in 70% alcohol, then preserved in several changes of 96% ethanol and stored at 4 °C. Air-dried specimens were transferred into 96% ethanol and also stored at 4 °C.

For each specimen, the locality, collection dates, altitude and habitat were noted. Table 3 lists the taxa for which partial sequences of the mitochondrial 16s rRNA gene were determined.

Additional sequences of *Agathemera crassa* (Blanchard, 1851) and the lentulid species *Karruia* sp., *Lentula obtusifrons*[1] Stål, 1878 and *Eremidium* nr. *equuleus* (Karsch, 1896) (selected from the NCBI GenBank) were chosen to root the phylogenetic trees.

Identification. Lentulidae species were identified using the keys of Jago (1981). The material was checked again against holotypes or paratypes of the Natural History Museum, London, as well as against material of the entomological collections of the National Museums of Kenya, Nairobi, and the Naturkunde Museum, Berlin.

[1]Found in NCBI GenBank as *L. obtusa*. However, this species name does not exist and we assume that the species investigated by Rowell & Flook (2004) was *L. obtusifrons*.

DNA extraction, amplification, purification and sequencing

For all specimens listed in Table 3, muscle tissue of the hind femur was used. Genomic DNA was extracted using different procedures: The QIAamp® DNA mini kit (Qiagen, Germany), following the standard protocol for blood and tissue, and the NucleoSpin® tissue kit (Machery & Nagel, Germany) following the standard protocol for human and animal tissue.

The extracted genomic DNA was used as a template for polymerase chain reaction (PCR) amplification with insect primers modified for Saltatoria (16a: 5′-CGC CTG TTT ATC AAA AAC AT-3′ and 16b: 5′-CCG GTC TGA ACT CAG ATC ACG T-3′; Kocher *et al*., 1989). Amplification was performed under the following conditions: initial denaturation 5 min at 94 °C; 38 cycles of 45 s at 94 °C, 45 s at 52 °C, 80 s at 72 °C, and a final extension step at 72 °C for 7 min.

Amplification product (2 μL) was loaded on to 1.5% agarose gels for size fractionation in gel electrophoresis to detect the amplification results (Sambook *et al*., 1989). A purification of amplification products was performed using the standard protocol of the QIAquick PCR purification kit (Qiagen).

Sequencing of the purified PCR products was conducted in-house on a Licor DNA 4200 IR2 automated sequencer using the thermo sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, Germany) and on an ABI Prism 377 sequencer (Applied Biosystems, U.S.A.) using the BigDye Ready Mix (Applied BioSystems). Additionally sequencing was partly outsourced (Sequencing Service, Macrogen, Korea).

Sequence analysis and phylogenetic reconstruction

Sequence data were edited using the program SEQMAN 4.03 (DNAStar). A basic local alignment search tool (BLAST; Altschul *et al.,* 1990) search at the NCBI GenBank (www.ncbi.nlm.nih.gov) served to detect potential contamination. The alignment was generated using the program CLUSTAL x (Thompson *et al*., 1997) and was subsequently corrected by eye. To optimize the alignment it was compared with the secondary structure of *Drosophila melanogaster* (GenBank no. X53506, comparative RNA web site, www.rna.icmb.utexas.edu; Gutell *et al*., 1994).

Table 2. Species of the genus *Usambilla*.

Original description	Jago (1981)	Distribution
Usambilla olivacea Sjöstedt	*U. turgidicrus olivacea* (Sjöstedt)	Tanzania, West Usambara
Lentula turgidicrus Karsch	*U. turgidicrus turgidicrus* (Karsch)	Kenya, Kitui, Taita Hills, Athi river; Tanzania, South Pare
Usambilla affinis Kevan & Knipper	*U. affinis affinis* Kevan & Knipper	Tanzania, Uluguru Mts.
	U. affinis kikomboensis Jago	Tanzania, Mpwapwa
Adolfia insolita Rehn	*U. insolita* (Rehn)	Zaire, Lake Kivu
Adolfia sagonai Ramme	*U. sagonai sagonai* (Ramme)	Zaire, Lakes region
	U. sagonai fractolineata Jago	Uganda, Mpanga forest reserve
	U. chlorophrygana Jago	Tanzania, Mpwapwa
	U. emaliensis Jago	Kenya, Emali Range
	U. haematogramma Jago	Tanzania, Ufipa plateau
	U. leptophrygana Jago	Tanzania, Dodoma
	U. oraria Jago	Kenya, Mombasa; Tanzania, West Usambara

To detect phylogenetic relationships, different methods of tree reconstruction were used: distance, maximum likelihood and maximum parsimony. The consistency of clades was tested by bootstrapping with 1000 replications and random addition of taxa using a heuristic search algorithm. All analyses were performed with PAUP* V4.0 (Swofford, 2003).

Using the dataset, the program MODELTEST 3.7 (Posada & Crandall, 1998) determined the best fitting model of sequence evolution for the model-depending algorithms selected by the Akaike information criterion (Akaike, 1974, 1976). The parameter estimates and likelihood scores were obtained for each model using PAUP* V4.0. The optimal model identified by MODELTEST 3.7 was used for distance and maximum likelihood analyses. For reconstruction using maximum parsimony assumptions, different procedures, such as the Fitch (1971) and the Carmin & Sokal (1965)

Table 3. Locality data for the specimens sequenced. Specimens were sequenced repeatedly to detect intraspecific variation among the sequences. All sequences are submitted to the NCBI Genebank.

Species	Locality	NCBI GenBank
Alitusambilla modicicrus (Karsch, 1896)	Mt. Kilimanjaro / Kifufu estate	DQ523690
Alitusambilla modicicrus (Karsch, 1896)	Mt. Kilimanjaro / Kidia	DQ523691, −2
Alitusambilla modicicrus (Karsch, 1896)	Mt. Kilimanjaro / Machame	DQ523693, −4, −6, −7
Alitusambilla modicicrus (Karsch, 1896)	Mt. Meru	DQ523695
Alitusambilla keniensis (Hemp, 2007*)	Mt. Kenya / Chogoria route	DQ523698, −9
Rhainopomma magnificum Jago, 1981	South Pare / Chabaru	DQ523721, −2
Rhainopomma magnificum Jago, 1981	South Pare / Mt. Shengena	DQ523719
Rhainopomma magnificum Jago, 1981	South Pare / Kisiwani	DQ523720
Rhainopomma montanum (Kevan, 1950)	Taita Hills / Wundanyi area	DQ523712−16
Rhainopomma pseudomontanum Hemp, 2007*	West Usambara / Kwagoroto forest	DQ523711, −17, −18
Rhainopomma uguenoensis Hemp, 2007*	North Pare / Mt. Kindoroko	DQ523704−8
Rhainopomma uguenoensis Hemp, 2007*	North Pare / Kiverenge Hill	DQ523702, −03, −09, −10
Rhainopomma usambaricum (Ramme, 1929)	East Usambara / Armani Kwamkoro	DQ523700−1
Usambilla affinis affinis Kevan & Knipper, 1961	Uluguru Mts. / Morningside	DQ523730−1
Usambilla olivacea Sjöstedt, 1909	West Usambara / Mombo	DQ523727−8
Usambilla olivacea Sjöstedt, 1909	Mt. Kilimanjaro / Weru Weru	DQ523723, −4, −9
Usambilla olivacea Sjöstedt, 1909	North Pare / Lembeni	DQ523725
Usambilla sp.	Manyara region/ Mbulu District	DQ523732−3
Usambilla turgidicrus (Karsch, 1896)	Mt. Kilimanjaro / Lume River	DQ523726
Usambilla turgidicrus (Karsch, 1896)	Taita Hills / Wundanyi area	DQ523734
Usambilla sagonai (Ramme 1929)	(Rowell & Flook 2004)	AY569279
Karruia sp.	(Rowell & Flook 2004)	AY569244
Lentula obtusifrons Stal, 1878	(Rowell & Flook 2004)	AY569242
Eremidium nr. *equuleus* (Karsch, 1896)	(Rowell & Flook 2004)	AY569243
Agathemera crassa (Blanchard, 1851)	(Flook & Rowell 1997)	X53506

*Hemp *et al.* (2007).

algorithm were tested and different substitution matrices were used.

For screening the quality of the generated genetic dataset, several statistic tests were performed: a χ^2-test to analyse the homogeneity of base frequencies, as well as the substitution saturation and the transition/transversion ratio to detect multiple substitution. For a graphical presentation of the calculated phylogenetic trees, the programs TREEVIEW 1.6.6 (Page, 2001) and MEGA 3.1 (Kumar *et al*., 2004) were used.

Results

Sequence data

The edited alignment included 499 characters, of which 225 were constant, 125 variable but parsimony uninformative and 149 parsimony informative. Analysing the nucleotide composition, a homogenous base distribution was assumed, supported by a χ^2-value of approximately 49.68 ($P = 1.0$). The sequences showed patterns typical of many insects, such as a high A–T content (72%) and variable distance-dependent transition/transversion ratios (Simon *et al*., 1994). The model selected with MODELTEST 3.7 was the transversional model with a gamma shape distribution (+ Γ) and invariant sites (I). The values of the estimated parameters were as follows: nucleotide frequencies (A) 0.3330, (C) 0.0980, (G) 0.1709, (T) 0.3981; substitution rates (A–C) 0.8047, (A–G) 4.2322, (A–T) 1.7317, (C–G) 0.3942, (C–T) 4.2322, (G–T) 1.0000; gamma shape parameter $\Gamma = 1.1677$; $P_I = 0.3008$.

Comparing the p-distances with the corrected d-distances, no significant substitution saturation caused by multiple substitutions was detected within the three lentulid genera (Fig. 3A). Saturation was found with increasing genetic distances, described by an increasing incongruity between data points and the diagonal line. This saturation described the comparison of the ingroup taxa with the outgroups.

Plotting the number of transitions and transversions against the d-distances gave similar results (Fig. 3B). A low ratio of transitions to transversions indicated multiple substitutions within the data (Wägele, 2005). This was only found in comparisons with the outgroup taxa.

Phylogenetic reconstruction

Phylogenetic trees calculated with different tree constructing methods and variation in assumptions on sequence evolution resulted in almost identical topologies. *Agathemera crassa* (Phasmatodea) was used to root the topologies. The cladograms are well resolved and the main splits are supported by high bootstrap values. Figure 4 shows the combined results obtained with different methods.

All investigated species of the three lentulid genera, *Altiusambilla*, *Rhainopomma* and *Usambilla*, defined by Jago (1981) proved to belong to monophyletic groups. At least for this set of species, generic discrimination was confirmed. *Usambilla* is the sister group of *Rhainopomma*, whereas *Altiusambilla* turned out to be a separate clade.

Usambilla olivacea collected from various localities along the Pangani river system turned out to be a separate species from *U. turgidicrus* found in the Kenyan highlands, the Taita Hills, the southern slopes of the Eastern Arc mountains and on the eastern slopes of Mount Kilimanjaro. Both groups are characterized by a pairwise genetic distance of maximum 0.4% within and above 4% between groups. Morphological differences between the two species *U. olivacea* and *U. turgidicrus* were seen in different measurements. *Usambilla olivacea* is more stocky and shows a different colour pattern than *U. turgidicrus*. Also, habitat seems to differs for the two species. Whereas *U. olivacea* is restricted to riverine forest communities along the southern slopes of the northern Eastern Arc chain and Mount Kilimanjaro, *U. turgidicrus* is found throughout the highlands of Kenya extending its area to the Taita Hills, the northern slopes of the South Pare mountains and also occurs on the eastern side of Mount Kilimanjaro. This

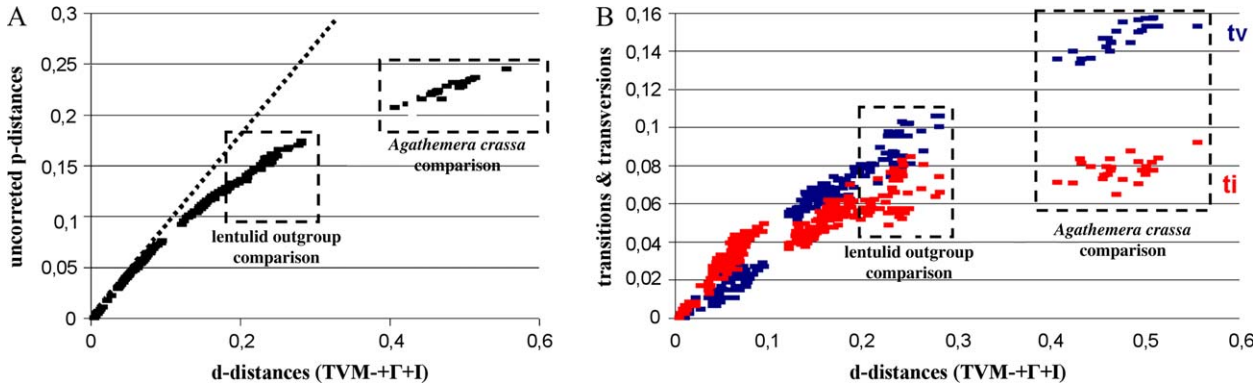

Fig. 3. A, Corrected d-distances plotted against the observed d-distance values; B, transitions and transversions plotted against the corrected d-distances.

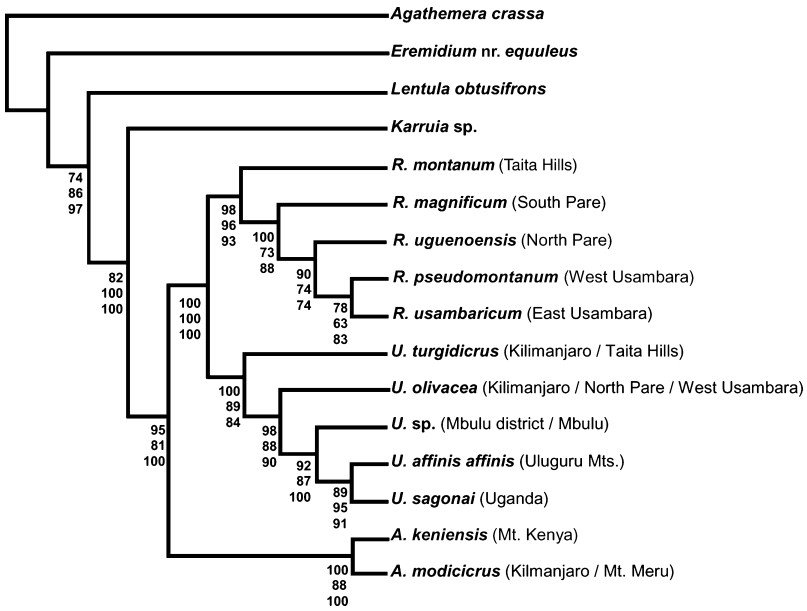

Fig. 4. Combined results obtained with different reconstructing methods; bootstrap values (1000 replicates) at nodes in the order: distance, maximum likelihood, maximum parsimony.

species also obviously occurs in drier habitats such as *Commiphora* and *Acacia* woodland, spiny vegetation on lava, scrub woodland and plantations (Jago, 1981), whereas *U. olivacea* hitherto was found only in moister riverine forest communities. Thus, it is proposed to re-establish the original name *U. olivacea*.

Usambilla affinis affinis was described from the Uluguru mountains near Morogoro from a locality named Morningside by Kevan & Knipper (1961) . This locality was visited in 2004 and material was collected (Table 3). *Usambilla sagonai* data were taken from GenBank. Because Rowell & Flook (2004) stated that the sample they investigated was collected in Mpanga forest, Uganda, the gene sequence incorporated here comes from the subspecies *U. s. fractolineata* (see Jago, 1981), which is here proposed as a sister taxon to *U. affinis affinis* from the Uluguru mountains of Tanzania. Because *Usambilla* species distributed geographically between the above-named taxa were not investigated in this paper (e.g. *U. chlorophrygana*, *U. leptophrygana*, and *U. haematogramma*), phylogenetic relationships might turn out differently when new species are investigated.

An as-yet undescribed species was collected on montane grasslands of the Mbulu District of Tanzania near the district city Mbulu. Genetic screening confirmed the status of these specimens as members of the genus *Usambilla*, as indicated by morphology and habitat. Until now only two specimens of this new species have been collected and description awaits more material becoming available.

The sister taxon of *Usambilla* turned out to be the genus *Rhainopomma*, as suggested by Jago (1981) due to the very similar morphology of species in the two genera. Jago (1981) differentiated this genus from *Usambilla* and all other lentulids by an extreme apical position of the male genitalic aedeagal barbs. In difference to *Usambilla*, *Rhainopomma* species occupy habitats with higher rainfall, mostly submontane to montane forest areas. Furthermore, this genus is restricted to the Eastern Arc mountains and coastal areas of Tanzania and Kenya. Five species of *Rhainopomma* were described by Jago (1981), of which three are investigated in this paper. *Rhainopomma uguenoensis* Hemp (Hemp *et al*., 2007) was collected in the North Pare mountains. As already suggested by their distribution, *Rhainopomma pseudomontanum* Hemp (Hemp *et al*., 2007) from the West Usambara mountains, placed under *R. montanum* by Jago (1981), turned out to be a separate species. Geographically, *R. montanum* from the Taita Hills is separated from the *Rhainopomma* species of other Eastern Arc mountains such as Pare and Usambara, which is also reflected in the molecular relationships.

Rhainopomma magnificum occupies a broad altitudinal span from moist colline to montane forests in the South Pare mountains. Although morphologically clearly distinct, *R. usambaricum* and *R. pseudomontanum* from the West Usambara mountains are sister species. *Rhainopomma usambaricum* is found in moist colline forests in an altitudinal range of approximately 400–1000 m in the East Usambara mountains, whereas today *R. pseudomontanum* is restricted to montane forest communities (e.g. Mazumbai forest reserve, Kwagoroto forest reserve). The North Pare mountains harbour an endemic species, *R. uguenoensis* (Hemp *et al*., 2007), which forms the sister group to the species from the East and West Usambara mountains (Fig. 4).

As already suggested by Jago (1981), morphological resemblance of *Rhainopomma* and *Altiusambilla* is a result of convergent evolution. Especially females of *Altiusambilla modicicrus*, *R. pseudomontanum* from the West Usambara mountains and *R. uguenoensis* from the North Pare mountains resemble each other astonishingly, as all have pronotal crests. The genus *Altiusambilla* is a separate lineage.

Molecular data proved that the species collected in montane forest on Mount Kenya belongs to *Altiusambilla*. This genus was until now monotypic, with the species *Altiusambilla modicicrus*, which occurs in the submontane and montane zones of the volcanoes Mounts Meru and Kilimanjaro and the Monduli Range of Tanzania. With *Altiusambilla keniensis* Hemp (Hemp *et al*., 2007) a second species of this genus has been found.

Discussion

The highest diversity of Lentulidae is found in southern Africa (Dirsh, 1965). Only seven of the 21 genera of the subfamily Lentulinae have their centre of diversity in tropical East Africa and some neighbouring countries. However, they comprise approximately one-half of all species. Arrays of closely related species occur on adjacent, today climatically isolated, high mountains of East Africa, suggesting recent radiation processes. Climatic fluctuations enlarging and diminishing forest cover and thus connecting nowadays isolated mountainous areas with each other with Lentulidae suitable habitats are also known to have happened several times, for example during the last ice age, as indicated by lake level changes (e.g. Bergner *et al*., 2003) and ice core borings on Mount Kilimanjaro (Thompson *et al*., 2002). That, for example *Rhainopomma* species are neo-endemics is also seen in their comparatively close molecular relationship.

It is assumed that when ancestors reached East Africa, a rapid speciation occurred due to new habitats and unoccupied niches. This assumption is prompted by the fact that all three investigated genera – *Altiusambilla*, *Rhainopomma* and *Usambilla* – are related to each other but are distinctly separated from South African taxa such as *Karruia*, *Eremidium* and *Lentula*, as already reported by Rowell & Flook (1998).

The spread of lentulids from South Africa might have been favoured by highland ranges. Ecologically adapted to climatic conditions prevailing in southern Africa, similar ecological niches were found at higher altitudes further north. Genera distributed at higher elevations between South Africa and East Africa are, for example *Basutacris* and *Qachasia* (Zimbabwe, Zambia), *Paralentula* (Zimbabwe) and *Nyassacris* (Malawi). When lentulids 'reached' tropical zones they adapted to moister and warmer conditions and ecological niches were filled, for example in montane forests, by for example *Rhainopomma* species. *Usambilla* species hereby represent a type that dwells at higher open-land altitudes but also developed species at lower altitudes (e.g. *U. turgidicrus* or *U. olivacea*). However, most *Usambilla* species occur on highlands. From ancestors shared with *Usambilla*, *Rhainopomma* might have evolved adapting to montane forest habitats in the area of the Eastern Arc range of Tanzania and even occupying coastal niches (e.g. *R. wapugu*).

Little can be said about *Altiusambilla*. At present only two species of *Altiusambilla* are known. This might, however, be the result of insufficient collection activity. Because *Altiusambilla modicicrus* inhabits the Mounts Meru–Kilimanjaro area in the south [for ecological data on *Altiusambilla modicicrus*, see, e.g. Hemp & Hemp (2003) and Hemp (2005)], and *Altiusambilla keniensis* Hemp is found as far as Mount Kenya in the north, it may be presumed that species of this genus might also be found in between.

Investigation of additional taxa combined with further collection efforts and studies about their ecology might illuminate migration corridors and speciation modes of East African Lentulidae.

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